6. Imaging the complexity, plasticity and dynamics of caveolae

Asier Echarri and Miguel A. Del Pozo

Integrin Signaling Laboratory, Vascular Biology and Inflammation Department, Centro Nacional de Investigaciones Cardiovasculares (CNIC), Melchor Fernández Almagro, 3, 28029, Madrid, Spain

Correspondence should be addressed to M.A.d.P, email: madelpozo@cnic.es

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6.1 Introduction

Communication between a cell and its environment is mostly mediated through the signals received and processed at the plasma membrane. The plasma membrane therefore needs to be able to sense and sort these signals, and in order to achieve this is organized into domains that accommodate specific populations of resident membrane molecules. Plasma membrane domains can be composed of specific lipids, such as lipid rafts [1], proteins [2, 3] or a combination of both. Caveolae are plasma membrane domains with a specific lipid and protein composition [4] that confers them with a characteristic membrane curvature, defining them as flask-shaped inward

plasma membrane invaginations with a diameter of 60-80 nm (Fig. 1). These membrane microdomains were first noticed in 1953 by Nobel laureate microscopist G.E. Palade and were described and named “caveola intracellularis” by E. Yamada in 1955. It took about 40 years to identify caveolin1 (Cav1), the main structural component of caveolae [5], followed a decade later by the discovery of a second family of proteins, the cavins, which have emerged as key regulators of caveolae formation and stability [6-8]. Now, after 60 years of research, we are just beginning to understand the physiology of caveolae, and the advent of new imaging tools is revealing the secrets of these enigmatic membrane invaginations.

6.2 Caveolae and their formation requirements

6.2.1 Caveolins, the main component of caveolae

The main constituent proteins of caveolae are proteins of the caveolin family. There are three mammalian caveolin genes: Cav1, caveolin-2 (Cav2) and caveolin-3 (Cav3). Cav1 and Cav2 are expressed in most cell types except skeletal muscle, whereas Cav3 is restricted to muscle. Cells of mice lacking Cav1 or Cav3 in their respective tissues do not have caveolae [9-11], strongly suggesting that Cav1 and 3 are the main essential caveolae components. Cav2 contributes to caveolae formation in some cell types but is not needed in vivo [4]. The abundance of caveolins in mammalian cells is highly variable, with some cell types, such as lymphocytes, neurons and hepatocytes, expressing Cav1 at low level, while cells in mechanically stressed tissues express Cav1 (adipocytes, fibroblasts, endothelial cells) or Cav3 (muscle) at high abundance [4]. Caveolins are restricted to metazoans, and are thus absent from fungi, plants and non-metazoan parasites [12]. Some organisms, such as *C. elegans*, express caveolin but do not contain caveolae [12]. While expression of honeybee caveolin in mouse cells devoid of caveolae is able to form caveolae, no caveolin gene has been identified in the fruit fly [12]. Expression of Cav1 in bacteria was recently

shown to induce formation of caveolae-like invaginations, suggesting that Cav1 is able by itself to bend the membrane to some extent [13].

Cav1 is an integral membrane protein that is post translationally modified by the addition of palmytic acid on three cysteine residues [14]. Cav1 is inserted in the membrane through a central region with a putative hairpin structure, leaving both ends of the protein in the cytosol [4]. Cav1 oligomerizes, and an estimated 144 Cav1 molecules can incorporate into a single caveolae [15]. The ability of Cav1 to bind cholesterol [16] and fatty acids [17] indicates a role in lipid biology. Many proteins have been shown to interact with Cav1, many of them through the scaffolding domain [18], although this view has been challenged [19, 20]. Cav1 binding proteins include membrane receptors, ion channels, adaptors, kinases and other signaling molecules, although many of these interactions may be indirect. Cav1 also interacts with proteins such as filamin A that link it to the actin cytoskeleton [21]. Some protein interactions are dependent on phosphorylation of Cav1 on tyrosine 14 [4].

6.2.2 Caveolae regulatory molecules

In the early phase of caveolae research attention focused exclusively on Cav1, mainly because of the absence of caveolae in Cav1 knockout mice. But subsequent studies provided a richer view of the players involved in the formation and regulation of caveolae. A proteomics screen for molecules abundant in caveolae identified PTRF (polymerase I and transcription release factor), SDPR (serum deprivation response, also known as sdr) and SRBC (sdr-related gene product that binds to C-kinase) [7]. These proteins were later renamed cavin1, cavin2 and cavin3 [8, 22], and a fourth cavin, cavin4 (also called Murc), was identified in muscle cells [23, 24]. These molecules form the cavin complex that is important for the stability of caveolae [25]. The functions of caveolins and cavins are highly interdependent. Cavin1 is required for the stability and

expression of all the other cavins and of caveolins, while Cav1 is required for the stability and expression of all cavins except for cavin4 [26]. In contrast, cavins 2 and 3 do not contribute to the stability of other known caveolar residents [26]. Cavin1 stabilizes Cav1 at the plasma membrane by facilitating its oligomerization and preventing its degradation [26, 27]. Cavin1 reaches the plasma membrane after Cav1 [28] and can be released from this location upon osmotic swelling [29], suggesting that cavin1 has roles unrelated to its participation in caveolae; indeed, cavin1 was originally isolated as a direct transcriptional regulator in the nucleus [30]. Analysis of cavin2 knockout mice showed that cavin2 is required for caveolae formation in lung endothelium and adipose tissue, but is dispensable in the endothelium of the heart. In contrast, caveolae in cavin3 knockout appear to be normal, at least structurally [26]; however, analysis of cavin3 silenced cells revealed a role in the trafficking of Cav1-positive spots [31].

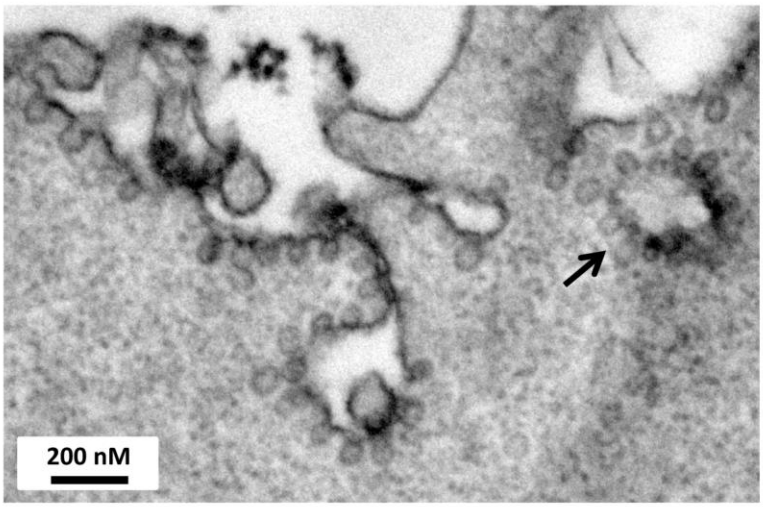
Although the biochemical details of the interactions between the cavin complex and caveolins or other caveolar residents are still unknown, cavin1 appears to interact with Cav1 and directly with cavin2 [32], and therefore seems to be more important for caveolae formation than the other members of the complex. Furthermore, cavin1 is the only cavin to induce caveolae biogenesis in PC3 prostate cancer cells, which express Cav1 but not cavins, and recruits the other members to caveolae [23]. Protein cross-linking before cell lysis allowed the immunopurification of a larger complex containing Cav1, Cav2 and cavins 1, 2 and 3 in a constant proportion independent of the immunotargeted protein. The most abundant protein in this complex is Cav1/2, followed by cavin1 —four times less— and cavin2 and 3, which compete with each other for binding to the complex [25]. This complex has been proposed to form the core that determines the shape of caveolae [25]. Cavins may also be important for the recruitment of signaling molecules to caveolae. Purified cavin2 in the presence of phophatidylserine binds PKCα, a regulator of caveolae

endocytosis, and cavin2 is required for the caveolar localization of this enzyme, suggesting that cavin2 may recruit proteins to caveolae [6]. Together, these studies suggest that cavin1 is essential, together with Cav1, for the formation of caveolae and the stability of key caveolae regulators [27], while the other cavins appear to play regulatory roles.

Other proteins that localize to caveolae or regulate its organization and trafficking properties include some that are able to shape membranes and tubulate them under specific conditions. For example, pacsin2, an F-BAR protein involved in clathrin-mediated endocytosis [33], localizes to a pool of caveolae, and plays a role in shaping them [34, 35]. Other studies provide evidence suggesting that the mobility of caveolae is dependent on EHD2, an ATPase that binds and tubulates the plasma membrane by oligomerization [36, 37]. This ATPase localizes to a pool of caveolae and prevents the mobility of Cav1 by favoring its anchorage to stress fibers by an unknown mechanism [38, 39]. In endothelium and some other tissues, the neck of caveolae is capped by the stomatal diaphragm, a structure formed by plasmalemmal vesicle associated protein (PV1) [40, 41].

Caveolar membrane domains are highly complex, and caveolae can be present as single units or groups, depending on the cell type and conditions. When several caveolae are interconnected they are referred as racesome invaginations, caveolar rosettes or simply rosettes (Fig. 1A arrow, B and 3). The molecules responsible for this plasticity are not known, but Abl kinases and mDia1-regulated actin fibers have been shown to impinge on this organization [42].

A



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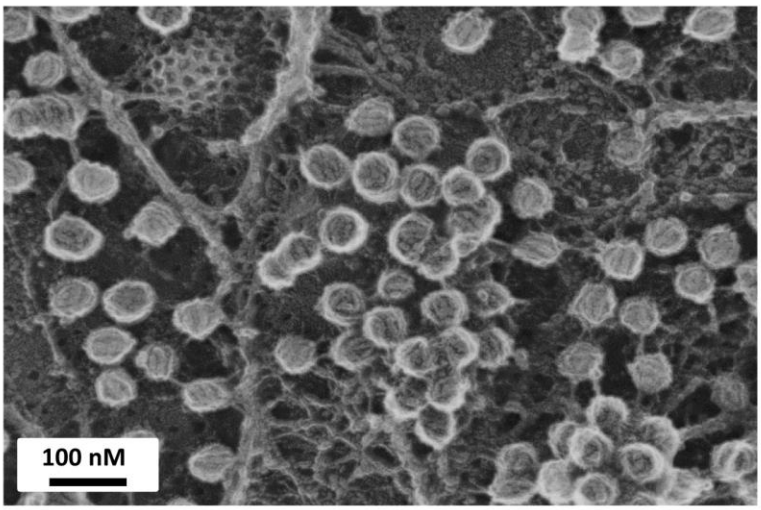

Figure 1
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6.3 Caveolae trafficking

6.3.1 Exocytosis and recycling of caveolae

Caveolae are formed exclusively at the plasma membrane and are not observed in endomembranes. However, newly synthesized Cav1 forms a complex in the Golgi, where it associates with cholesterol and forms a precursor of caveolae [43, 28]. After reaching the plasma membrane, cavins and possibly other factors are recruited and contribute to the formation of caveolae [28]. Exocytosis of caveolae is dependent on SNAP23 and syntaxin4 [44], and other factors required for membrane fusion have been identified in caveolae [45]. Upon loss of cell adhesion, Cav1 accumulates in the Rab11 positive recycling endosomes and upon readhesion recycles back to the plasma membrane in a process tightly regulated by integrins [46-48]. In this pathway, integrin-linked kinase, mDia1 and IQGAP regulate the transport of Cav1 vesicles to the plasma membrane in a microtubule-dependent manner [49]. The recycling of Cav1 upon cycles of de-adhesion and adhesion has also been shown to be regulated by the exocyst component Exo70, actin and microtubules [50].

6.3.2 Endocytosis of caveolae

The endocytosis of caveolae is difficult to analyze for various reasons. First, under basal conditions, endocytosis of caveolae is infrequent [51]. Second, there is no known specific cargo of caveolae to facilitate tracking, since cargoes assigned to caveolae, such as cholera toxin B subunit or SV40, can enter Cav1-deficient cells due to their ability to enter through other routes [52, 51]. Finally, surface-connected caveolae can be observed deep in the cytosol, frequently in groups referred as rosettes, making it difficult to distinguish endocytosed caveolae from membrane folds containing caveolae (Fig. 1A arrow and 3) [53]. For these reasons, many studies

have tracked the inward trafficking of membrane receptors or Cav1 itself and have characterized the involvement of Cav1 in the trafficking of particular receptor or the involvement of factors in the regulation of Cav1 inward trafficking. Several membrane residents have been shown to traffic in a Cav1-dependent manner, suggesting that they are endocytosed through caveolae. Multiple studies link Cav1 with TGFβ signaling [54, 55], and TGFβ receptor itself has been shown to traffic through caveolae under certain conditions, which negatively regulates its stability [56]. Similarly, Wnt signaling uses caveolae as a regulatory platform in the trafficking of LRP6, a subfamily of low-density-lipoprotein (LDL) receptor related proteins. Stimulation of LRP6 with Dkk1 induces its endocytosis through clathrin-mediated endocytosis, while stimulation with Wnt3a redirects it to caveolae, resulting in different signaling outputs [57], so that Wnt3a induced β-catenin translocation to the nucleus is dependent on Cav1 [58]. Caveolae are highly abundant in the endothelium and various studies show a role of caveolae trafficking in this tissue. Endothelin, a potent vasoconstrictor, induces endocytosis of the endothelin receptor type B through caveolae [59]. The role of caveolae in transcytosis in the endothelium has been debated for many years [60-62] but the presence of caveolae in tubulovesicular structures penetrating deep into the cytosol might favor this type of specialized transport. A role for caveolae in tissue permeability has been linked to its ability to endocytose membrane proteins such as occludin [63] and E-cadherins [64].

The trafficking properties of caveolae are highly dependent on integrins. Integrins not only regulate the recycling of Cav1 [48] but also, under specific conditions, regulate caveolae endocytosis [65]. β1Integrins and fibronectin are themselves endocytosed in a Cav1-dependent manner in some cell types [66-68] but not in others [69]. A recent paper showed that syndecan-4–stimulated cells induce α5β1 integrin endocytosis in a Cav1- and RhoG-dependent manner [70]. Interestingly, RhoG was previously implicated in caveolae endocytosis [71]. The trafficking of

Cav1 to the perinuclear area upon loss of cell adhesion is highly dependent on actin filaments and microtubules [42], as is Cav1 endocytosis induced by other stimuli [72, 73]. Regulators of stress fibers, including Abl tyrosine kinases, mDia1 and filamin A regulate this process [42, 47, 74]. Caveolae entry is also regulated by the tyrosine kinase Src [75, 76]. The Abl and Src kinases phosphorylate Cav1 on tyrosine 14, a residue involved in trafficking [48, 77]. Integrin signaling and the stress fiber regulatory machinery thus appear to play a key role in controlling the plasma membrane Cav1 pool. Similar to clathrin-mediated endocytosis, caveolae endocytosis requires dynamin2 [47, 78, 76, 79, 75, 42]. Shortly after the identification of caveolin-1, a role was identified for PKCα in caveolae endocytosis, and this has since been corroborated [47, 80]. PKCα phosphorylates filamin A and c-Abl, key regulators of caveolae trafficking [47, 81]. Caveolae endocytosis is also triggered by other stimuli, including hyperosmotic medium and okadaic acid [73], mitosis [82] and certain viruses [83-85]. Furthermore, the stability of caveolae at the plasma membrane is also strongly influenced by the membrane lipid composition [75, 76].

6.4 Visualization of caveolae

6.4.1 Electron microscopy (EM)-based technology to study caveolae morphology

The most obvious characteristic of caveolae is their shape, and since this can only be identified unambiguously by electron microscopy, caveolae can only be unequivocally identified using EM techniques (Fig. 1). Even though this morphological criterion is generally accepted, given the relatively constant and unique diameter and shape of caveolae, in some cases additional immunolabeling with caveolin antibodies may be required. The precise shape of caveolae differs depending on the EM technique used, ranging from flask-shaped in glutaraldehyde-fixed samples to open cups, without a clear constricted neck, in cryofixed samples [86]. It is important to take these differences into account for visualization and analysis.

Circular vesicles with a caveolar diameter can frequently be observed in the cytosol, (Fig. 1A and 3), and immunolabeling with Cav1 may be needed to assign a caveolar origin if a caveolar shape is not clear. Tomography, the 3D reconstruction from thin serial sections, has been successfully employed to define the surface connection of these internal caveolae-like vesicles and the complexity in the organization of caveolar domains [87-89]. The surface connection of these internal caveolar structures can be established by the use of cell non-permeable labels such as ruthenium red during fixation [42, 53] or by using membrane impermeable quenching agents, such as ascorbic acid, targeted against HRP bound to cholera toxin [51]. This last approach, using anti-Cav1 specific immunogold labeling, detected the budding of a small fraction of clustered and what appear to be individual caveolae [51]. However, individual caveolae were not detected by tomography, so it is possible that the apparently individual caveolae detected by immunogold labeling are part of clusters centered outside the section plane.

Freeze-fracture immunolabeling of plasma membrane Cav1 in unfixed cells shows that Cav1 concentrates towards the neck of the caveolae [90], a finding supported by scanning EM in combination with tungsten-labeling [91]. These findings suggest that the neck region might be a functionally distinct subdomain within caveolae [92]. However, analysis of carbon-platinum fast-freeze, deep-etch replicas by immunoelectron microscopy showed Cav1 staining in the caveolar bulb [5], and a recent study using nano-gold-conjugated secondary antibodies revealed a caveolar bulb distribution of Cav1, cavin1, 2 and 3 [25]. The coating of the caveolar bulb appears striated or spiked depending on the EM protocol used (Fig. 1B)[93, 94]. Although the exact protein composition of this structure is not fully determined, it appears to include Cav1 [5, 89, 88, 95]. A recent study used a mini singlet oxygen generator (mini-SOG) approach to study the distribution of caveolar components. This technique is based on the ability of an engineered fluorescent

flavoprotein from *Arabidopsis* to generate reactive oxygen upon illumination; the oxygen radicals catalyze the polymerization of diaminobenzidine, producing a dark, electrodense precipitate used to localize the protein by EM [96]. Fusion constructs of the mini-SOG with the protein of interest allow its identification with better spatial resolution than classical immunogold techniques because of the small size of the flavoprotein compared with the antibodies used in immuno-EM techniques. Using this approach, Nichols and co-workers showed that caveolae are coated by a structure, the caveolar coat complex, likely consisting of Cav1, Cav2 and cavins 1, 2 and 3 [25]. Dual-tilt tomograms and miniSOG EM reveal that this structure is repeated with a constant spacing over the surface of the caveolar bulb [25].

The link between caveolae and the actin cytoskeleton was suggested by the first antiserum raised against caveolin, which revealed caveolae decorating actin cables [5]. This was also observed by quick-freeze, deep-etch studies on rat aortic endothelium [97]. Similarly, electron tomography of the cytoplasmic surface using rapidly frozen, deeply etched, platinum-replicated plasma membranes showed that 93% of caveolae are associated with the actin filaments of the membrane skeleton, similar to the proportion of actin-associated clathrin coated pits [93]. A tight link between the caveolar system and the cytoskeleton was confirmed by studies of fast-frozen/freeze-substituted cells and immunolabeled plasma membrane lawns, together with analysis by 3D electron tomography, and these studies also revealed a heterogeneous organization of caveolae [89].

The potential of correlative light and electron microscopy (CLEM) has yet to be full exploited in the caveolae field. To the best of our knowledge, three studies have used this approach. A study of exocytosis measured SNAP23- and syntaxin4-mediated fusion of caveolar vesicles with the plasma membrane and showed that the presence of SNAP23 correlates with different vesicle

fusion states [44]. A recent study used immunofluorescence techniques to show cavin3 is enriched together with Cav1 at the rear of migrating cells, and EM analysis of this pattern revealed multiple caveolae, rosettes and caveolae-like vesicles at the plasma membrane and penetrating relatively deep into the cell body [25], once again demonstrating the ability of caveolae to form complex structures. Immunostaining has also showed an association of Cav1 with curved caveolae in human placenta samples [98]. The resolution of caveolae structures observed by light microscopy at the EM level will be highly informative.

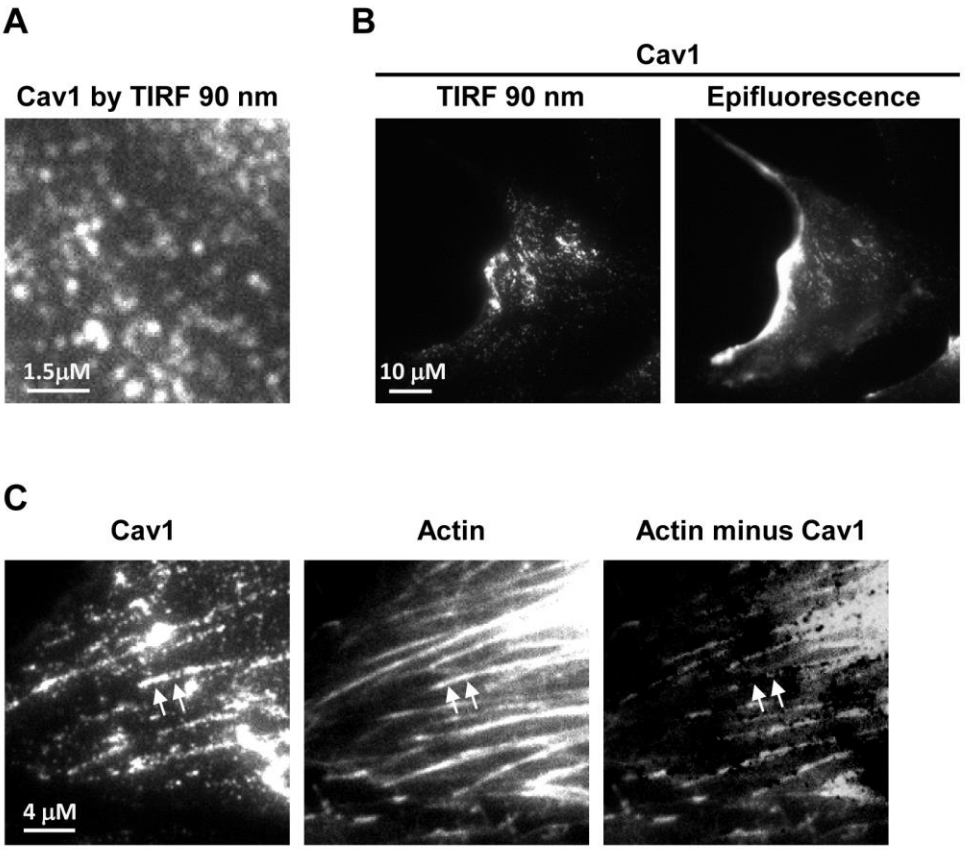

Figure 2
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6.4.2 Analysis of caveolae by epifluorescence, confocal and total internal reflection fluorescence microscopy (TIRF-m)

The Z-resolution in epifluoresence and confocal microscopy does not differentiate caveolae at the plasma membrane from internal vesicles, but these techniques have nonetheless provided valuable information about the motility and organization of caveolae and Cav1. Confocal fluorescence microscopy has been universally applied to study caveolin localization in cells, showing that Cav1 staining is concentrated in retracting areas of the cell (Fig. 2B), early or recycling endosomes, and co-aligned with stress fibers (Fig. 2C) [42, 47, 5, 85]. In epithelial cells, Cav1 accumulates at cell-cell junctions [99], whereas in transmigrating endothelial cells Tyr 14 phosphorylated-Cav1 accumulates in the forward extensions [100]. In muscle cells, Cav3 localizes to T-tubules [101]. The dynamics of caveolae have been studied indirectly by intravital fluorescence microscopy to investigate the role of caveolae in endothelial cells [102, 103]. Confocal microscopy, alone or in combination with fluorescence recovery after photobleaching (FRAP), has been used to study the dynamics of GFP-tagged Cav1 or other caveolar components, such as cavins or EHD2 [31, 104, 39]. Gervásio and colleagues used fluorescence resonance energy transfer (FRET) to indirectly study the flattening of caveolae upon membrane stretching. Exploiting the relative enrichment of caveolae with the sphingolipid GM1 [105], they used cholera toxin B subunit (which binds GM1) tagged with alexa-fluor 555 or 647 as FRET donor and acceptor, respectively. FRET efficiency increased upon Cav3 expression, suggesting that Cav3 reduces the distance between GM1 molecules. This was reversed by stretching, indicating increased separation, probably caused by flattening of caveolae [106]. A similar approach using fluorescence-lifetime imaging microscopy (FLIM)/FRET showed that the cavin complex is formed in the cytosol and associates with caveolae at the plasma membrane [23]. Image cross-correlation spectroscopy has been used to

study the colocalization of BMP receptors with Cav1-positive structures and their dynamic behavior after stimulation [107].

The complexity of caveolae is clearly revealed by the EM techniques described above and by TIRF-m (Fig. 1A, B and 2A). TIRF-m analysis of immunostained endogenous Cav1 or Cav1-GFP allows identification of the different Cav1-positive populations based on their fluorescence intensity in diffraction-limited fixed spots [15, 42]. Although this analysis only quantifies the "amount" of fluorescence in a defined region that correlates with the number of molecules, it allows comparison of the effects of a given treatment on the organization of caveolar domains. This analysis reveals a quite large diversity in caveolar structures, correlating with the diversity observed by EM techniques (Fig. 1A and 2A). However, TIRF-m cannot be used to define or count the number of caveolae since flattened Cav1-positive domains —flattened caveolae— co-exist with invaginated caveolae [5] and many caveolae can fit into a diffraction-limited spot. In addition, the dimmer spots detected by this technique could represent internal Cav1 spots that are further from the TIRF plane and are not necessarily caveolae. Despite these limitations, TIRF-m is a powerful tool for studying caveolae organization and dynamics. Using TIRF-m, the dynamic behavior of plasma membrane proximal Cav1 spots can be precisely followed in the xy and z axes [15, 47, 42]. The so-called kiss-and-run movement of Cav1-GFP observed by TIRF-m is not sensitive to dynamin2, Abl kinases or mDia1, all regulators of Cav1 inward trafficking [42]; however, this movement is slightly sensitive to cytochalasin D (Echarri A and Del Pozo MA, unpublished observations), suggesting that different caveolar pools exist. Flattening of caveolae was suggested by TIRF-m, although this required confirmation by EM and immuno-EM analysis [29].

6.4.3 Super-resolution microscopy applied to caveolae

The use of super-resolution microscopy has begun to be applied in the caveolae field and has been used to describe other plasma membrane invaginations, such as clathrin-coated pits [108]. A study using fluorescence photo-activation localization microscopy (FPALM) showed that CRFB1, a subunit of the zebrafish IFN-R (Type I interferon receptor) complex, colocalized with caveolin in clusters at the plasma membrane. Caveolin silencing reduced the numbers of these CRFB1 clusters [109], suggesting that caveolin forms clusters that condition the organization of other molecules. A super-resolution optical imaging approach using stochastic optical reconstruction microscopy (STORM) showed that Cav3 in mouse cardiac myocytes is present in different sized clusters colocalized with ryanodine receptors [110]. Diffraction limited resolution indicated 28.6% colocalization between these molecules, whereas super-resolution showed a colocalization of just 4.9%, suggesting that optical blurring was responsible for the higher colocalization observed by diffraction limited resolution [111]. Dual-color super-resolution was also used to observe different-sized Cav1 spots labeled with anti-GFP camelid nanobodies [112]. Stimulated emission depletion (STED) gives a significantly better resolution of Cav1 spots than confocal microscopy [113], reaching around 128 nm in cells expressing low levels of Cav1. These spots are probably Cav1 scaffolds, since they were significantly smaller than the average diameter of spots in cells expressing normal levels of Cav1 [114]. STED has also been used to study the response of Cav3-positive domains in transverse tubules after myocardial infarction. Myocardial infarction significantly alters the distribution of Cav3, and STED revealed an increase in the number of Cav3-positive longitudinal structures between striations, concomitant with an increase in other longitudinal transverse tubule components [115]. However, the shape of caveolae in these structures could not be resolved [115, 113].

The further application of super-resolution microscopy will undoubtedly reveal new information about the structure of caveolae and the spatial relationship between caveolins and other caveolar components such as cavins, EHD2, pacsin2 and dynamin2. Super-resolution microscopy also has the potential to provide valuable information about the interplay between caveolae and filamin A, stress fibers and other cytoskeleton related molecules. It is unclear how this technology will visualize the different levels of caveolar curvature—flattened, curved or in a fission/fusion process—but if dynamic behavior is included, it will certainly increase our understanding of caveolae biology.

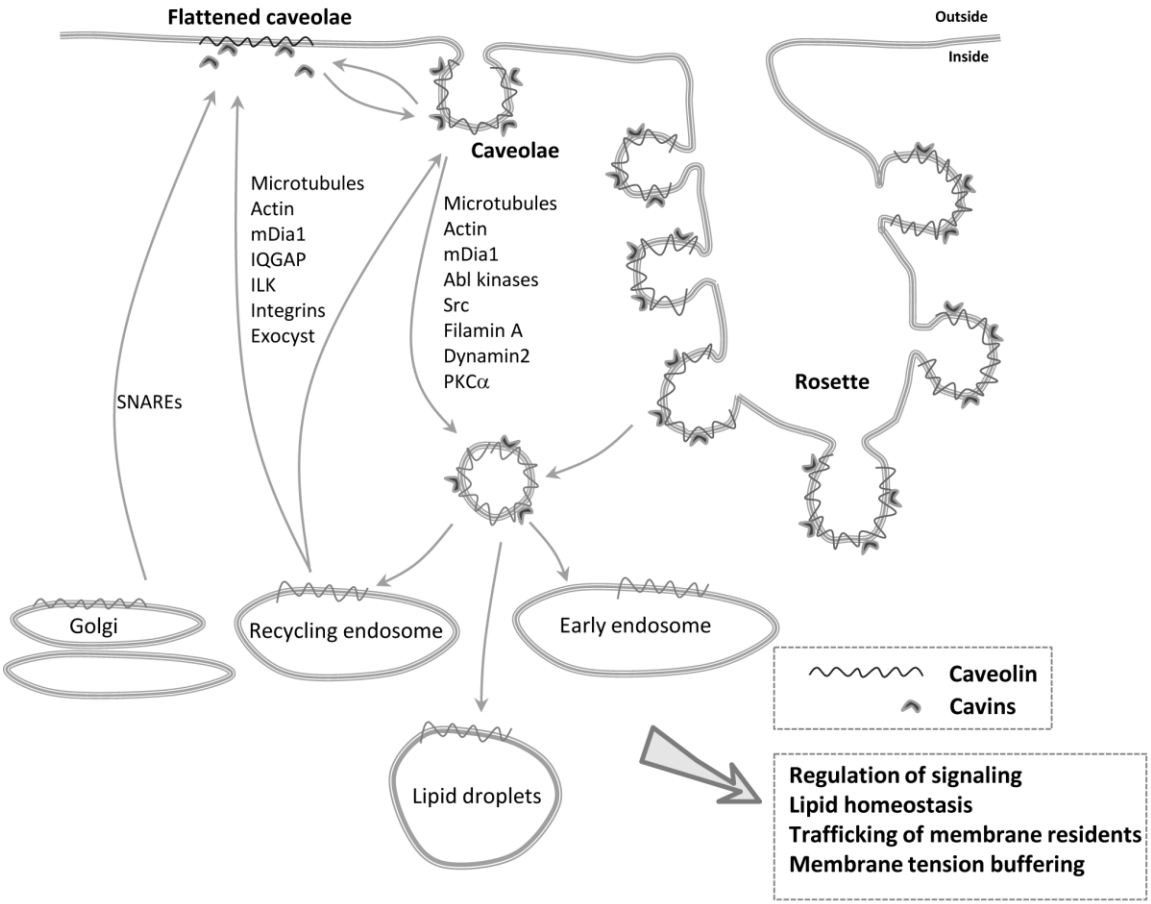

Figure 3
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6.5 Caveolar functions and human disease

Although mice lacking caveolae are viable and fertile, the presence of caveolae in cells provides certain advantages that facilitate optimal cell function [116]. The exact function of caveolae is still debated. The phenotypes of mice lacking Cav1, Cav3 or cavins 1-3 and of human patients with natural mutations provide important clues. The most marked effects of the absence of caveolae is observed in adipose and muscle tissue, where caveolae are highly abundant. The numerous other minor abnormalities of caveolin-deficient mice have been reviewed previously [116]. Patients with Cav1, Cav3 or cavin1 mutations have lipodystrophy, muscular dystrophies and, to a lesser extent, cardiac alterations. The first human mutations in Cav3 were described in muscular dystrophy patients [117, 118] and various Cav1 mutations have been found in patients with lipodystrophy [119, 120]. Cav1 deficiency leads to less body fat and smaller and more fragile adipocytes [121-123]. The identification of cavin1 as key caveolae protein [124] was followed by reports of mutations in cavin1 in patients with lipodystrophy and muscular dystrophies [125-130]. Human cardiac syndromes associated with cavin1 mutations have also been reported [125, 131], and lack of cavin1 in humans and mice is linked to metabolic alterations [124, 128].

The role of Cav1 in regulating signaling pathways involved in proliferation, migration and adhesion indicated that Cav1 would be important in some stages of cancer progression [132-134]. Several studies showed that Cav1 acts as a tumor suppressor through its ability to block cell proliferation and metastasis [133-137]. In addition, several potent oncogenes induce the downregulation of Cav1, suggesting that Cav1 counteracts the action of these oncogenes [138]. Other studies, however, appear to show a role for Cav1 in tumor progression in melanoma, prostate and breast cancers [139-142]. The absence of cavins 1, 2 and 3 from some tumor samples also links the cavin family to cancer progression [143-146]. The complex involvement of caveolae components in tumor progression reflects the involvement of caveolae in multiple signaling

cascades and the complexity of cancer in terms of altered signaling, types and tumor stages and the interplay between stroma and tumor cells.

The general function of caveolae as platforms that regulate the emanation of signals from the plasma membrane is detailed above (Fig. 3). The shape of caveolae might be explained by its ability to endocytose, although endocytosis is uncommon and most caveolae retain a constant invaginated shape. The fact that caveolae can flatten in response to increased tension [29, 42, 147] suggests that caveolae could serve as membrane reservoirs that buffer the cellular adaptation to mechanical stress. This change in curvature would simultaneously modulate signaling for an appropriate response to this stress [147]. In this context, the pathologies associated with caveolae and the abundance of caveolae in mechanically stressed tissues and the diseases with which they are associated suggest that the regulation of mechanotransduction may be the principal function of caveolae [148].

Caveolae and Cav1 also appear to be important in lipid homeostasis. In Cav1 deficient cells, free cholesterol accumulates in mitochondria, resulting in susceptibility to apoptosis [149]. Cav1 localizes to lipid droplets and lipid droplet trafficking is disrupted by a dominant negative form of Cav1 [150]. Furthermore, the trafficking properties of Cav1 are regulated by the levels of cholesterol and fatty acids [151, 76] and the curvature of caveolae is dependent on cholesterol [5]. Caveolae are thus tightly linked to lipid biology. This, together with a mechanoprotective role of caveolae [121], could explain the lipodystrophy phenotypes of humans and mice lacking functional Cav1 genes. Imaging the structure, organization and movement of caveolae in vivo is still a challenge, but this approach could contribute to the understanding of the function of this important membrane curvature domain.

FIGURE LEGENDS

Figure 1. Electron microscopy images showing different organizations of caveolae

A) Mouse embryo fibroblasts were detached from the substratum and held in suspension for 20 minutes. Glutaraldehyde-fixed cells in the presence of ruthenium red, to label surface-connected structures, were embedded in epon and imaged by electron microscopy. Caveolae and a surface-connected rosette (arrow) are observed. **B)** Deep-etch replicas imaged by EM of differentiated 3T3-L1 adipocytes, revealing individual and clustered caveolae. A clathrin-coated pit is visible in the upper left corner. Produced in the Heuser laboratory by N. Morone and obtained from Krijnse Locker J, and Schmid SL (2013) PloS Biology, 11 (8): e1001639. Doi:10.1371/journal.-pbio.1001639.

Figure 2. Images of different patterns of endogenous Cav1 distribution

A) NIH3T3 cells were stained for endogenous Cav1 and imaged by TIRF microscopy with a penetration of 90 nm. Spots of differing sizes and intensities are visible. **B)** Mouse adult fibroblasts were plated on fibronectin-coated dishes and imaged 24 h later. The same polarized cell was imaged by TIRF-m at 90 nm penetration or by epifluorescence (right image). The pool of Cav1 in the retracting area is only visible in the epifluorescence image, indicating that it is located in the upper part of the cell. **C)** Cav1 co-aligns with stress fibers. NIH3T3 cells were fixed and stained for Cav1 and actin with phalloidin alexa-rhodamine. In the right image the Cav1 image was subtracted from the actin staining, revealing the empty spaces on stress fibers corresponding to the alignment of Cav1 spots with stress fibers (arrows).

Figure 3. Caveolae: organization, trafficking regulators, and major Cav1/caveolae functions

Flattened caveolae, isolated caveolae and caveolae in rosettes are depicted. Known proteins

involved in endocytosis and exocytosis/recycling are indicated.

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